

CHARACTERIZATION AND APPLICATION OF TANNASE PRODUCED BY *ASPERGILLUS NIGER* ITCC 6514.07 ON POMEGRANATE RIND

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ABSTRACT

Extracellular tannase and gallic acid were produced optimally under submerged fermentation at 37 °C, 72 h, pH 5.0, 10 % (v/v) inoculum and 4 % (w/v) of the agroresidue pomegranate rind (PR) powder by an *Aspergillus niger* isolate. Tannic acid (1 %) stimulated the enzyme production by 245.9 % while with 0.5 % glucose, increase was marginal. Tannase production was inhibited by gallic acid and nitrogen sources such as NH₄NO₃, NH₄Cl, KNO₃, asparatic acid, urea and EDTA. The partially purified enzyme showed temperature and pH optima of 35 °C and 6.2 respectively which shifted to 40 °C and 5.8 on immobilization in alginate beads. Activity of the enzyme was inhibited by Zn⁺², Ca⁺, Mn⁺², Mg⁺², Ba⁺² and Ag⁺. The immobilized enzyme removed 68.8 % tannin from juice of aonla/myrobalan (*Phyllanthus emblica*), a tropical fruit, rich in vitamin C and other essential nutrients. The enzymatic treatment of the juice with minimum reduction in vitamin C is encouraging as non enzymatic treatments of myrobalan juice results in vitamin C removal.

Key words: tannase, pomegranate rind, myrobalan, *Aspergillus niger*

INTRODUCTION

The enzyme tannase (E.C 3.1.1.20) also known as tannin acyl hydrolase, is a hydrolytic enzyme that acts on tannin. Tannase catalyses the hydrolysis of bonds present in the molecules of hydrolysable tannins and gallic acid esters (8) producing gallic acid. Gallic acid is used in the food industry as a substrate for the chemical synthesis of food preservatives such as gallates and pyrogallol, propyl gallate being a very important food antioxidant, and in the pharmaceutical industry, for the synthesis of antibacterial drugs (16). The main commercial applications of tannase at present are in the

preparation of instantaneous tea, action on tea polyphenols, in the production of gallic acid (17), beer chillproofing and wine making. Tannase can also be used to reduce tannin levels wherever desirable such as fruit juices (14). Aonla (*Phyllanthus emblica* Linn.), a tropical fruit is one of the richest sources of vitamin C and has been recognized since ancient times for its immense medicinal and nutritional properties. This fruit with its high tannin content i.e. gallotannic acid, which upon hydrolysis yields gallic acid, has antioxidant properties and retards the oxidation of vitamin C. The stability of vitamin C in aonla products due to gallic acid makes its processing a matter of great concern.

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The astringent taste due to tannins of aonla juice on the other hand reduces its acceptance as a beverage. Hence enzymatic hydrolysis of tannin in aonla juice into gallic acid is advantageous since it reduces its astringency with minimum loss of vitamin C.

The novelty of the present work lies in the use of a new agro substrate pomegranate rind (PR) by an *A.niger* isolate for optimum production of gallic acid and tannase and application of the latter for the first time in tannin removal from juice of aonla, a nutrient rich tropical fruit without loss of its nutritional value.

MATERIALS AND METHODS

Isolation and screening of microorganisms

A fungal strain was isolated on Czapeck-Dox medium containing 1 % (w/v) tannic acid and selected from eight other microbial strains on the basis of zone of lysis and tannase activity (1). The strain was identified as *Aspergillus niger* by the Indian Type Culture Collection, New Delhi and deposited in their collection unit (*A.niger* ITCC 6514.07).

Inoculum preparation

The culture was maintained on tannic acid agar slants stored at 4 °C and subcultured at regular intervals of three weeks. For inoculum preparation the culture was grown at 37 °C for 7 days in Czapeck-Dox medium containing (g/l): NaNO₃ 6.0; KCl 0.52; MgSO₄.7H₂O 0.52; KH₂PO₄ 1.52; Cu (NO₃)₂.3H₂O traces; ZnSO₄.7H₂O traces; FeSO₄ traces supplemented with 4 % PR and the spores (5.0x10⁷) were scraped into 5 mL of the same medium and incubated at 37 °C for 24 h to inoculate 50 mL of fermentation medium.

Growth conditions

Tannase production was carried out on Czapeck-Dox medium supplemented with 4% PR as the sole carbon source. The pH of the medium was either 4.0 or 5.0 and 50 mL of the medium was sterilized in 250 mL of Erlenmeyer flask at 121 °C for 20 min cooled and inoculated with pre induced

inoculum and incubated at 37 °C at 220 rpm, other conditions remaining the same. Extracellular tannase and gallic acid were estimated in the fermented broth at an interval of 24 h. All the experiments were carried out in triplicates and the analyses were done in duplicates. Mean values are shown in the figures and the tables with error bars.

Preparation of substrate

Pomegranate rind was spreaded on trays and oven dried at 70 °C for 24 h. The dried rind was ground and sieved to obtain particle size of 425 µm and stored in polyethylene bags at room temperature (30 ± 5 °C).

Tannase assay

Extracellular tannase is being reported here as intracellular tannase was very negligible (data not shown). For assay of extracellular enzyme the aliquots of the fermented broth were withdrawn at desired time intervals, filtered and centrifuged and the supernatant was used as the extracellular enzyme. Tannase was assayed by the method based on chromogen formation between gallic acid and rhodanine (15). The reaction mixture containing 0.25 mL of 0.01 M methyl gallate in 0.05 M citrate buffer, pH 5.0 and 0.25 mL of extracellular enzyme was incubated at 30 °C for 10 min and 0.3 mL of methanolic rhodanine (0.667 % w/v) was then added. After 5 min. 0.2 mL of 0.5 M KOH was added. A control was run where enzyme was added after the addition of KOH. Finally the reaction mixture was diluted by 4.0 mL distilled water and incubated at 30 °C for 10 min and absorbance was recorded at 520 nm. One unit of tannase is the amount of enzyme which liberated 1 µmol of gallic acid in one minute.

Gallic acid estimation

Gallic acid was estimated in the fermented broth. To 0.5 ml of fermented broth, 0.3 ml of methanolic rhodanine was added followed by 0.5 M KOH and gallic acid content was estimated by the method described above using a calibration graph using (10µg- 50µg) of gallic acid.

Optimization of process parameters for tannase production using PR

Optimum tannase production was determined for incubation period (24 h-96 h), substrate conc. (1 %-5 %), temperature (30 °C-40 °C), pH (3.0-7.0) and inoculum size (5 %-15 %) v/v.

Enzyme Purification

The enzyme was partially purified by acetone precipitation followed by DEAE cellulose column chromatography. Tannase was eluted with 0.02 M acetate buffer of pH 5.0 (13). This enzyme was used for determining the kinetic parameters.

Estimation of tannin content

The tannin content in the aonla juice was measured following the protein precipitation method by tannins (5).

Immobilization of tannase

A 10 mL suspension containing 488 units of tannase and 4 % sodium alginate was extruded dropwise through a 2 mL syringe into a 0.2 M CaCl₂ solution at 4 °C to form beads of 0.4 mm diameter. After 2 h the beads were washed with water and either preserved in 0.2 M CaCl₂ solution at a temperature of 6 °C or used (6).

Juice Preparation

5.0 g of aonla fruit was taken, cut into small pieces and ground in a mortar pestle. Juice was then extracted by adding 100 mL of distilled water and homogenizing in a blender followed by filtration through muslin cloth. The extracted juice was stored at 4 °C for further use.

Use of immobilized enzyme for tannin removal in aonla juice

15 mL of aonla juice was treated in a 100 mL conical flask with 15 beads of alginate entrapped enzyme (36.6 units) at 37 °C with stirring. Aliquots of 0.2 mL from the treated juice were taken out at 60 min intervals for analysis of the

tannin content. The treatment was carried out for 180 min as the optimum enzyme concentration and incubation period were found to be 36.6 units and 180 min for tannin removal (data not shown). After completion of the process, the beads were taken out from the juice and washed with distilled water. These beads were reused with fresh juice for two more cycles and then washed and stored in CaCl₂ solution at 6 °C for further use.

Statistical analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried using student 't' test. Statistical analysis was performed using the statistical package for social sciences (SPSS for windows; SPSS Inc., Chicago, IL

RESULTS AND DISCUSSION

Optimization of process parameters using PR as substrate

Optimum tannase production (28.72 U/mL) and gallic acid (52 µg/mL) were achieved after 72 h with 4 % (w/v) substrate (PR), at 37 °C, pH 5.0 with an inoculum size of 10 % (v/v). The decrease in enzyme activity after 72 h may be due to reduced nutrient level of medium affecting the metabolic activity and enzyme synthesis, inhibition and denaturation of the enzyme (4). The isolated *A.niger* reported here produced maximum tannase of 28.72 U/mL and gallic acid 52 µg/mL at 37 °C whereas most other *A.niger* strains reported have an optimum temperature of 30 °C (7,12,15).

Effect of tannic acid on tannase production

Tannase is an inducible enzyme produced only in the presence of tannic acid or its end product. The effect of tannic acid concentrations ranging from 0.2 %-1.5 % in the fermentation medium was studied. The tannase activity was stimulated by 245.9 % with 1 % tannic acid (101.2 U/mL) whereas the tannase activity with 1.5 % tannic acid was also significantly higher (45.1 U/mL) compared to control value

(29.25 U/mL) shown in Fig. 1. This suggests that pure tannic acid induced and stimulated tannase production while tannin present in PR is in complex form and not as readily available as tannic acid.

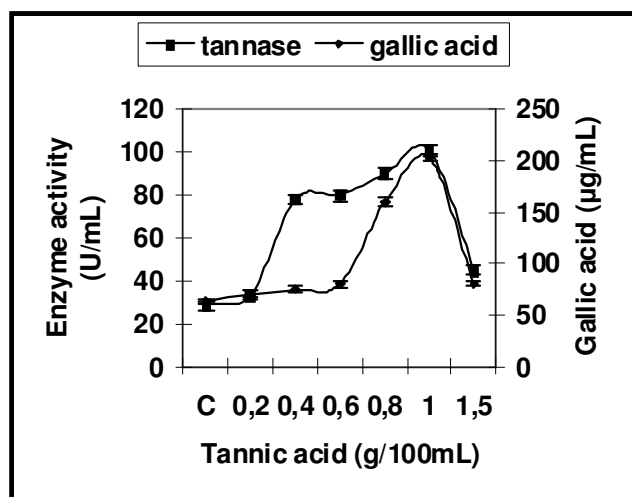


Figure 1. Effect of tannic acid on tannase and gallic acid production by *A.niger* isolate. T= 37 °C; S= 4 % (w/v); pH: 5.0; t= 72 h; I= 10 %(v/v). Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of P<0.05.

Effect of glucose on tannase production

The effect of glucose concentrations ranging from 0.25-1.0 % was studied which showed a marginal increase in tannase activity (30.8 U/mL) with 0.5 % of glucose as compared to control (29.25 U/mL) whereas the activity decreased with 0.75 % of glucose (Fig. 2). Similar observations were made where enzyme activity increased with 0.2 % of glucose while decreased with 0.05 and 0.5 % concentrations as compared to the control value (1). Glucose is one of the products of tannin degradation hence in higher concentrations it perhaps shows feed back inhibition.

Figure 3. Effect of different concentrations of gallic acid on tannase production.

t= 72 h, T= 37 °C, S= 4 % (w/v), pH= 5.0, I= 10 % (v/v). Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of P<0.05.

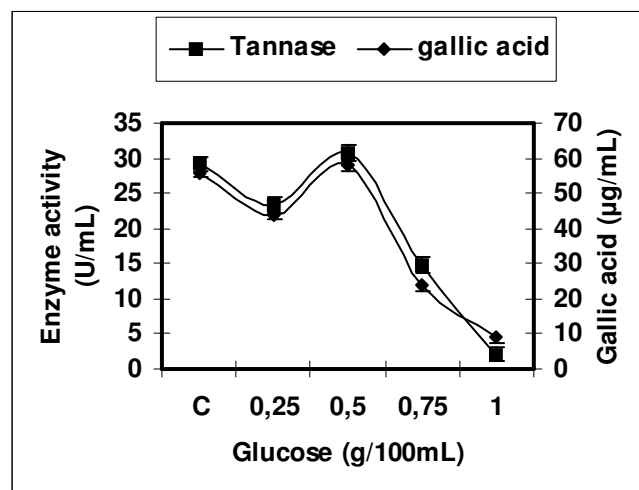
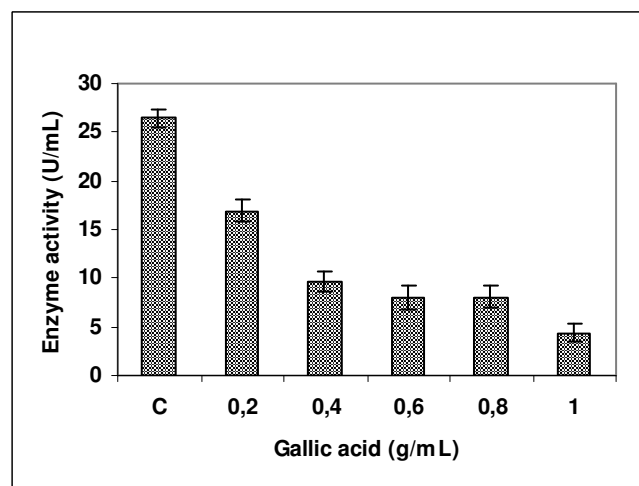


Figure 2. Effect of glucose concentrations on tannase and gallic acid production by *A.niger*.

t= 72 h, T= 37 °C, S= 4 % (w/v), pH= 5.0, I= 10 % (v/v). Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of P<0.05.

Effect of gallic acid on tannase production

Different concentrations of gallic acid ranging from 0.2-1 % when added to the production medium containing 4 % PR, showed inhibition (Fig. 3) which could be due to competitive inhibition. It has been reported that gallic acid, pyrogallol and gallaldehyde competitively inhibited the tannase of *A.niger* (9).



Effect of nitrogen sources and EDTA on tannase production

The effects of NH_4NO_3 , NH_4Cl , aspartic acid, urea, KNO_3 and EDTA on tannase production were studied. 0.2 % of these nitrogen sources were added to the production medium containing PR as substrate. All the nitrogen sources and EDTA acted as inhibitors for tannase production as compared to the control value where NaNO_3 was used as a nitrogen source (Fig.4).

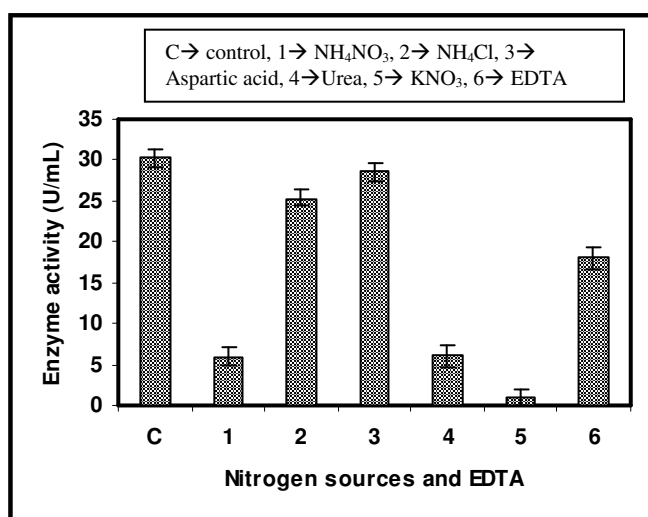


Figure 4. Effect of nitrogen sources and EDTA on tannase production by *A.niger* isolate.

$t= 72$ h, $T= 37$ °C, $S= 4$ % (w/v), $\text{pH}= 5.0$, $I= 10$ % (v/v), conc. of nitrogen sources and EDTA= 0.2%. Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of $P<0.05$.

Determination of kinetic parameters of tannase

The partially purified (soluble) enzyme was found to have an optimum temperature and pH of 35 °C and 6.2 respectively (Fig. 5 and 6). The K_m and V_{max} values of the latter were found to 0.012 mM and 33.3 $\mu\text{mole}/\text{min}$ from the LineWeaver Burk plot. The enzyme in immobilized state however had an optimum temperature of 40 °C and optimum

pH of 5.8 (Fig. 5 and 6). The elevation of temperature in case of immobilized enzyme is due to the entrapment of the enzyme which protects it from adverse heat effects and makes it thermally stable. A shift in the optimum temperature of immobilized enzyme has been reported in other cases also (11).

Effect of metal ions and nitrogen sources on tannase activity

The effect of metal ions such as ZnCl_2 , CaCl_2 , MnCl_2 , AgNO_3 , MgCl_2 and BaCl_2 on tannase activity were studied. A volume of 0.25 mL of partially purified enzyme was added to 0.25 mL of substrate (methyl gallate) containing 1.0 mM concentration of metal ions and incubated at 35 °C for 10 min. All the metal ions studied acted as inhibitors in which MnCl_2 showed minimum inhibition whereas CaCl_2 showed maximum inhibition (Fig. 7). The influence of different inorganic nitrogen sources like NH_4NO_3 , NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_4)_2\text{SO}_4 \cdot \text{FeSO}_4 \cdot 6\text{H}_2\text{O}$ in the reaction mixture for enzyme assay were also studied. NH_4Cl was found to stimulate the activity marginally while all the other nitrogen sources exerted inhibitory effects (Fig. 8).

Application of immobilized tannase for tannin removal from fresh aonla juice

Tannin removal by immobilized tannase in fresh aonla juice containing 1125 $\mu\text{g}/\text{ml}$ of tannin having pH of 2.5 was tested at 37 °C for 180 min. It can be seen that maximum hydrolysis of 68.8 % took place after 3 h in the first run (Table 1 & Figure 9). The beads were used for three successive runs resulting in 37.7 % and 24.4 % tannin hydrolysis after 2nd and 3rd run respectively. The results were encouraging considering the beads could be used successively for three runs (9 h) with 24.4 % tannin removal. Enzymatic treatments of fruit juices to reduce haze and bitterness have been reported by various authors (2, 3, 10). Reports on application of tannase in fruit juice processing are rare and there is no report of tannin removal from aonla juice which has a very strong astringency due to tannins. However

a 25 % reduction in tannin content from pomegranate juice by soluble tannase has been reported (14). Better performance of the immobilized enzyme used in this work is shown with 68.8 % tannin removal. After 3 h treatment of aonla juice with tannase there was a 2 % reduction in vitamin C content (Table 1) which was considerably low compared to

other conventional processing methods which involve non-enzymatic heat treatments and results in removal of vitamin C. Based on these findings further studies are being carried out as enzyme catalyzed detannification of aonla juice has not been reported so far and can be nutritionally beneficial.

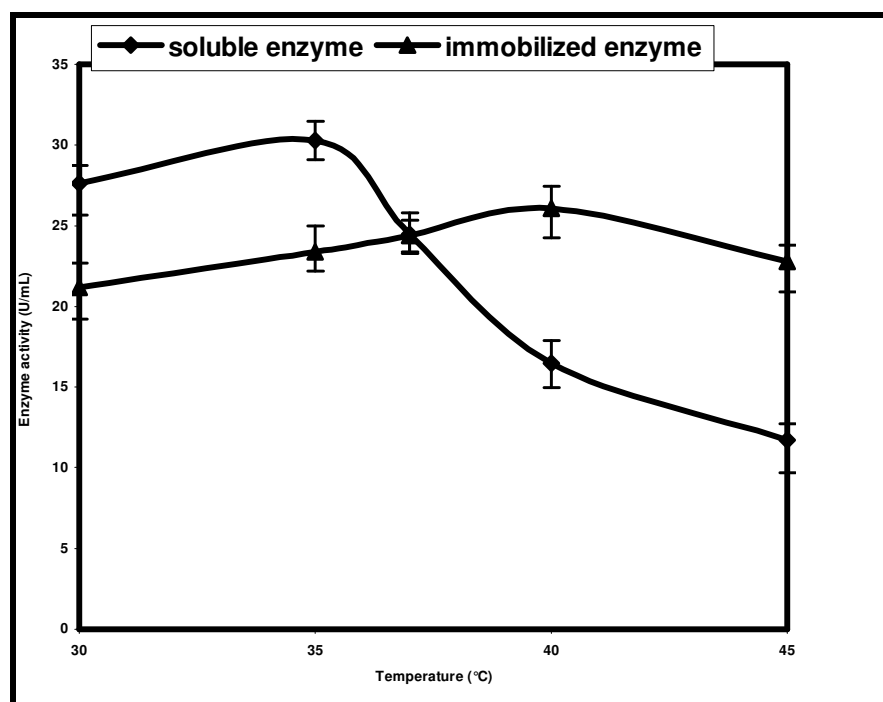


Figure 5. Determination of the optimum temperature of free and immobilized tannase by the isolated *A.niger* strain. Reactions were carried out for 10 min with 0.01 M methyl gallate substrate in 0.05 M citrate buffer, pH 5.0. Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of $P < 0.05$.

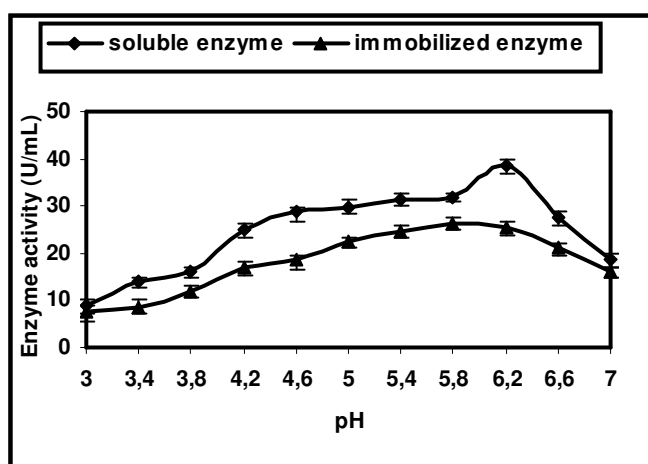


Figure 6. Determination of the optimum pH of free and immobilized tannase by the isolated *A.niger* strain. Reactions were carried out for 10 min with 0.01 M methyl gallate substrate in 0.2 M citrate phosphate buffer at 30°C, incubation period 72 h. Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of $P < 0.05$.

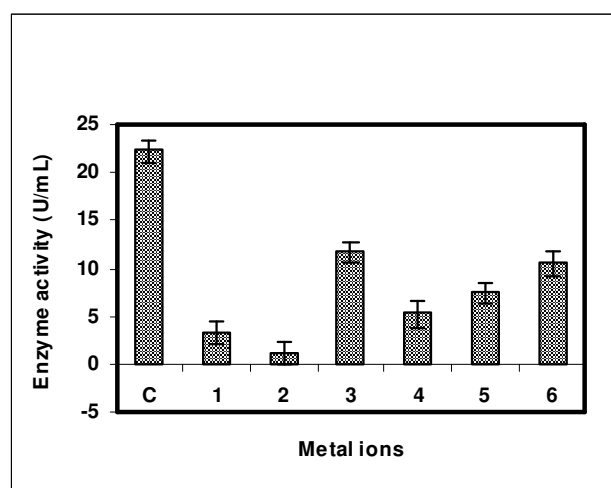


Figure 7. Influence of metal ions on tannase activity. Reactions were carried out for 10 min with 0.01 M methyl gallate substrate in 0.05 M citrate buffer, pH 5.0 containing 1.0 mM of metal ions. Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of $P < 0.05$.

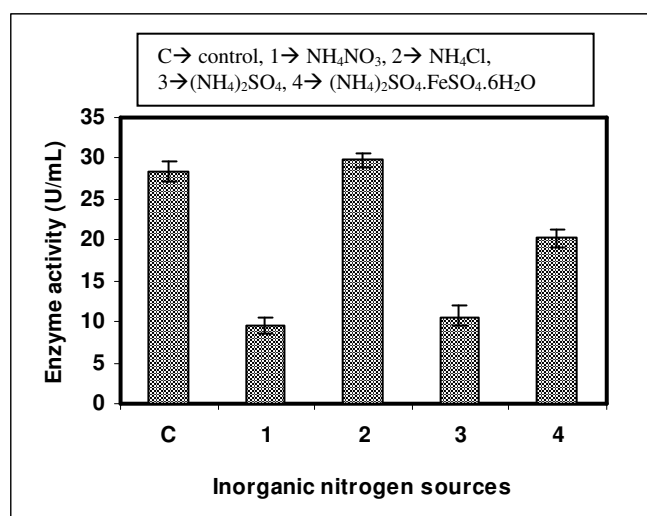


Figure 8. Effect of inorganic nitrogen sources on tannase activity. Reactions were carried out for 10 min with 0.01 M methyl gallate in 0.05 M citrate buffer, pH 5.0, containing 1.0 mM nitrogen sources. Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of $P < 0.05$.

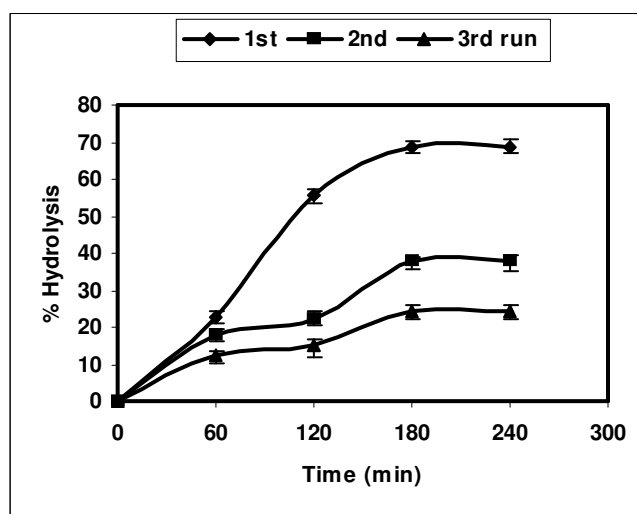


Figure 9. Effect of immobilized enzyme on hydrolysis of tannin after 1st, 2nd and 3rd run at 37 °C. The reactions were carried out with 15 beads containing 36.6 units enzyme and 15 ml of aonla juice. After each run of 180 min the beads were taken out, washed with distilled water and reused with 15 mL of fresh juice. Error bars indicate standard deviation from triplicate determinations. Data are mean of three independent readings with significance of $P < 0.05$.

Table 1. Tannin hydrolysis in aonla juice by immobilized enzyme as a function of time

Incubation period (min)	Tannin content (µg/ml)	*Tannin hydrolysis (%)	*Vitamin C content (mg/mL)	*Reduction in vitamin C content (%)
0	1125	-	1.00 ± .01	-
60	875	22.2 ± 1.1	0.995 ± 0.3	0.5 ± 0.3
120	500	55.5 ± 0.8	0.988 ± 1.1	1.2 ± 1.1
180	350	68.8 ± 0.7	0.980 ± 0.4	2.0 ± 0.3

*Mean of the three independent readings ± SD
 15 ml of juice was treated with 15 beads containing 36.6 units enzyme at 37°C, 150 rpm. 1.0 ml aliquot was withdrawn and analysed at 60 min interval for tannin hydrolysis, vitamin C and acidity levels.

CONCLUSIONS

Tannase produced by *A.niger* under optimized conditions on a novel agroresidue was partially purified and immobilized in alginate beads. The immobilized enzyme showed a higher temperature and lower pH optima than the soluble enzyme and could remove 68.8% tannin from

myrobalan juice in 180 min at 37°C, which has not been reported earlier.

REFERENCES

1. Bradoo, S.; Gupta, R.; Saxena, R.K. (1997). Parametric optimization and biochemical regulation of extracellular tannase from *Aspergillus japonicus*. *Proc. Biochem.*, 32(2),135- 139.

2. Chatterjee, S.; Chatterjee, S.; Chatterjee, B.P.; Guha, A.K. (2004). Clarification of fruit juice with chitosan. *Proc. Biochem.*, 39, 2229-2232.
3. Floribeth, V.; Lastreto, C.A. (2004). A study of the production of clarified banana juice using pectinolytic enzymes. *J. Food. Technol.*, 16, 115-125.
4. Gautam, P.; Sabu, A.; Pandey, A.; Szakacs, G.; Saccol, C.R. (2002). Microbial production of extracellular phytase using polystyrene as inert solid support. *Bioresour. Technol.*, 83, 229-233.
5. Haggerman, A.E.; Butler, L.G. (1978). Protein precipitation method for determination of tannins. *J. Agric. Food Chem.*, 26, 809- 812.
6. Kierstan, M.; Bucke, C. (1977). The immobilization of microbial cells, subcellular organelles and enzymes in calcium alginate gels. *Biotech. Bioeng.*, 19, 387-397.
7. Lekha, P.K.; Lonsane, B.K. (1994). Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid state, liquid surface and submerged fermentation. *Proc. Biochem.*, 29, 497-503.
8. Lekha, P.K.; Lonsane, B.K. (1997). Production and application of tannin acyl hydrolase; state of the art. *Adv. Appl. Microbiol.*, 44, 215-260.
9. Mahadevan, A.; Sivaswamy, S.N. (1985). Tannins and microorganisms. In *Frontiers of Appl. Microbiol.* eds K.G Mukherji, N.C. Pathak and V.P. Singh. Rastogi and company, Meerut., India 327-347.
10. Mishra, P.; Kar, R. (2003). Treatment of grapefruit juice for bitterness removal by amberlite IR 120 and amberlite IR 400 and alginate entrapped naringinase enzyme. *J. Food Sci.*, 68 (4), 1229-1233.
11. Park, N.H.; Chang, H.N. (1979). Preparation and characterization of immobilized naringinase on porous glass beads. *J. Ferm. Technol.*, 57, 310-306.
12. Pourrat, H.; Regeat, F. Pourrat, A.; Jean D. (1982). Production of tannase (Tannin acyl hydrolase E.C. 3.1.1.20) by a strain of *Aspergillus niger*. *Biotechnol. Lett.*, 4(9), 583- 588.
13. Rajakumar, G.S.; Nandy, S.C. (1983). Isolation, purification and some properties of *Penicillium chrysogenum* tannase. *Appl. Environ. Microbiol.*, 46(2), 525- 527.
14. Rout, S.; Banerjee, R. (2006). Production of tannase under mSSF and its application in fruit juice debittering. *Ind. J. Biotechnol.*, 5, 346-350.
15. Sharma, S.; Bhat, T.K.; Dawra, R.K. (2000). A spectrophotometric method for assay of tannase using rhodanine. *Anal. Biochem.* 279 (1), 85- 89.
16. Sharma, S.; Gupta, M.N. (2003). Synthesis of antioxidant propyl gallate using tannase from *Aspergillus niger van Tiegham* in non aqueous media. *Bioorg. Med. Chem. Lett.*, 13(3), 395-397.
17. Vardin, H.; Fenercioglu, H. (2003). Study on the development of pomegranate juice processing technology: clarification of pomegranate juice. *Nahrung.*, 42, 300-303.